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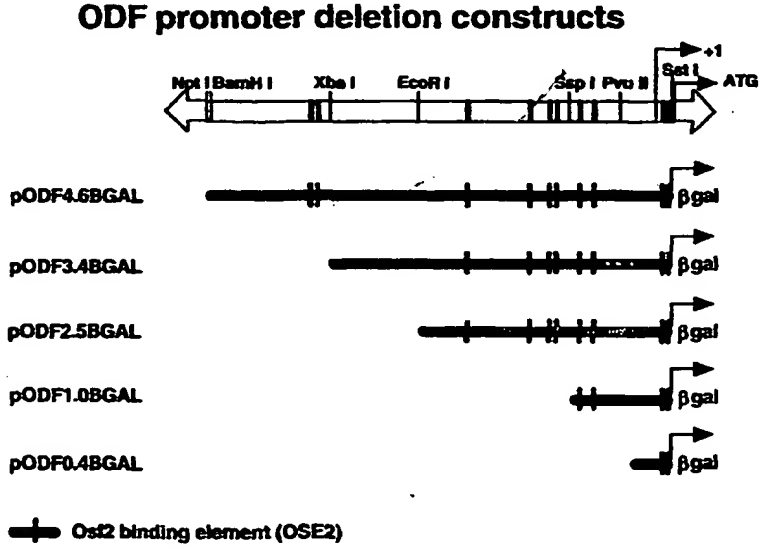
(54) Title: OSTEOCLAST DIFFERENTIATION FACTOR REGULATORY REGION



(57) Abstract: The present invention provides the complete transcriptional regulatory region of the human *odf* gene. The disclosed sequence, fragments thereof, and functional variants thereof, can be used in methods for regulating osteoclastogenesis, and treating bone diseases and other diseases caused by over- or under-expression of osteoclast differentiation factor. The disclosed sequences are also useful in diagnosing patient susceptibility to developing ODF-related bone, cartilage, immune, and arterial diseases, and for diagnosing patient receptivity to treatment with drugs for such diseases. Methods for identifying compounds that modulate osteoclast formation, bone resorption, and other ODF-related bone, cartilage, immune, and arterial diseases are also provided.

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Also provided is a computer based method for performing diagnosis, comprising determining the nucleotide sequence of the ODF regulatory region of DNA from a human subject, and comparing this sequence to a nucleotide sequence comprising, consisting essentially of, or consisting of the ODF regulatory region nucleic acid sequence shown in SEQ ID NO:1, or fragment thereof useful for diagnostic purposes, in a computer readable medium to identify any polymorphism or mutation that may be present in said human subject's DNA.

C. **THERAPEUTIC METHODS**

In another embodiment, the present invention provides methods of modulating osteoclast formation and function, bone resorption, and the other diseases, symptoms, and conditions discussed herein. As used herein, the term "modulate" or "affects" denotes an alteration, i.e., either an increase or a decrease. Thus, for example, a compound that modulates bone resorption is one that either increases or decreases bone resorption. A compound that "affects" reporter gene expression is one that stimulates or inhibits reporter gene transcription or expression. In the present context, compounds that stimulate or increase ODF gene expression are referred to as "agonists," while those that inhibit or decrease ODF gene expression are referred to as "antagonists." Osteoclast formation and function, and therefore bone resorption, can be modulated by administering to a patient one or more compounds identified by the methods described herein. For example, a compound that decreases reporter gene expression in a screening assay of the present invention employing a nucleic acid construct comprising the present ODF regulatory region, fragment thereof, or functional variant of either, is expected to be a candidate for treatment of abnormal bone resorption; osteoporosis; arterial disease; metastatic bone disease such as that resulting from prostate cancer, breast cancer, multiple myeloma, humoral hypercalcemia of malignancy, and lung cancer; rheumatoid arthritis;

osteoarthritis; Paget's disease of bone; hypercalcemia of malignancy; osteolysis; and periodontal disease. A compound that inhibits reporter gene expression, and hence ODF expression, would be expected to be effective, for example, for treating or preventing osteoporosis, a condition characterized by decrease in bone mass with decreased bone density, mineral content, and connectivity, producing porosity and fragility; tumor metastasis to bone; and rheumatoid arthritis. A compound that stimulates or increases reporter gene expression in a screening assay would be expected to increase ODF expression, and hence be effective for preventing or treating osteopetrosis, a condition characterized by abnormal thickening and hardening of bone. Similarly, immune responsiveness or function, including, for example, lymph node development, T- and B-cell development, T-cell activation, etc., can be regulated by administering to a patient one or more compounds identified in the methods described herein. As discussed above, since osteoclasts are derived from hematopoietic precursors, alterations in osteoclast precursor proliferation or differentiation can directly or indirectly affect immune modulation, lymph node development, and T- and B-cell formation.

Alternatively, gene therapy can be utilized by administering to a patient a pharmaceutical composition comprising a recombinant DNA construct comprising the ODF regulatory region disclosed herein, a fragment thereof, or a functional variant thereof, operably linked to the *odf* gene. The literature teaches a variety of different methods for introducing exogenous genes into cells *ex vivo* and *in vivo*; vectors for delivering nucleic acids can be viral, non-viral, or physical. See, for example, Rosenberg et al., *Science*, 242:1575-1578 (1988), and Wolff et al., *Proc. Natl. Acad. Sci. USA*, 86:9011-9014 (1989). Recent reviews discussing methods and compositions for use in gene therapy include Eck *et al.*, in *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, Ninth Edition, Hardman *et al.*, eds., McGraw-

Hill, New York, (1996), Chapter 5, pp. 77-101; Wilson, *Clin. Exp. Immunol.* 107(Suppl. 1):31-32 (1997); Wivel et al., *Hematology/Oncology Clinics of North America, Gene Therapy*, S.L. Eck, ed., 12(3):483-501 (1998); Romano et al., *Stem Cells*, 18:19-39 (2000), and the references cited therein. U.S. Patent No. 6,080,728 also provides a discussion of a wide variety of gene delivery methods and compositions. The routes of delivery include, for example, systemic administration and administration *in situ*. Well-known viral delivery techniques include the use of adenovirus, retrovirus, lentivirus, foamy virus, herpes simplex virus, and adeno-associated virus vectors. Exemplary non-viral techniques include the use of naked DNA; DNA complexed with cationic lipids, alone or in combination with cationic polymers; anionic and cationic liposomes; DNA-protein complexes and particles comprising DNA condensed with cationic polymers such as heterogeneous polylysine, defined-length oligopeptides, and polyethylene imine, in some cases contained in liposomes; and the use of ternary complexes comprising a virus and polylysine-DNA. Physical methods include the use of needle-free injectors, such as "gene gun" devices and devices using liquid under high pressure for delivery into interstitial spaces, and electroporation.

Administration of pharmaceutical preparations comprising the present ODF regulatory region or fragments thereof disclosed herein, or functional variants thereof, can be systemic, such as with liposomes, by, for example, intravenous injection. Specific expression of constructs in target cells can occur predominantly from the specificity conferred by the cell type-specific expression due to the ODF regulatory sequence, or this regulatory sequence in combination with the nucleic acid delivery vehicle targeting particular cell types. Administra-tion can also be *in situ*, such as with viral vectors. Delivery of recombinant constructs can be limited by localized introduction, for example by catheter (see U.S. Patent No. 5,328,470), local injection, or by stereotactic

injection (Chen et al., *Proc. Natl. Acad. Sci. USA*, 91:3054-3057 (1994)).

Suitable vectors can be constructed by any of the methods well known in the art. See, for example, Sambrook et al., *Molecular Cloning, a Laboratory Manual*, Second Edition, Cold Spring Harbor Press (1989), and Ausubel et al., eds., *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1987 and updates). The use of cationic liposomes, such as the DC-Chol/DOPE liposome, has been widely documented as an appropriate vehicle to deliver DNA to a wide range of tissues through intravenous injection of DNA/cationic liposome complexes. See Caplen et al., *Nature Med.*, 1:39-46 (1995); Zhu et al., *Science*, 261:209-211 (1993). Liposomes transfer genes to target cells by fusing with the plasma membrane. Examples of the successful application of liposome complexes include those of Lesson-Wood et al., *Human Gene Therapy*, 6:395-405 (1995), and Xu et al., *Molecular Genetics and Metabolism*, 63:103-109 (1998).

Pharmaceutical compositions for gene therapy comprising ODF regulatory region constructs can comprise the desired nucleic acid delivery system, and a pharmaceutically acceptable carrier, diluent, or excipient. Such compositions can also be used in transfecting cells for *in vitro* assays such as those described herein. Slow release matrices containing the nucleic acid delivery vehicle can also be employed. Where desirable or necessary, the delivery system can comprise a pharmaceutical composition comprising recombinant cells, and a pharmaceutically acceptable carrier, diluent, or excipient.

For use in the assay, diagnostic, and therapeutic methods disclosed herein, the present invention also provides in one of its aspects a kit or package, in the form of a sterile-filled vial or ampoule, that contains a polynucleotide comprising SEQ ID NO:1, a fragment thereof, or a functional variant thereof, or a vector containing SEQ ID NO:1, etc., operatively linked to the *odf* gene or a heterologous coding

sequence such as a reporter gene or other polynucleotide, as well as instructions for use in these various methods. The vector can optionally be contained within a vector-releasing cell. In one embodiment, the kit contains a polynucleotide vector containing an ODF regulatory region, fragment thereof, or functional variant thereof, operatively linked to an *odf* coding region as an administration-ready formulation, in either unit dose or multi-dose amounts, wherein the package incorporates a label or manual with instructions for use of its contents for the treatment of one or more of the symptoms, conditions, or diseases discussed herein. In another embodiment, the package provides a sterile-filled vial or ampoule containing a vector-releasing cell or cell line. Such kits or packages can also contain media and reagents, such as reaction buffers, for carrying out appropriate methods as disclosed herein with the nucleic acids, recombinant constructs, vectors, or cells contained therein, as well as instructions therefor.

From a prophylactic or therapeutic point of view, any prevention or alleviation of an undesirable symptom, condition, or disease as noted herein would be desirable. Thus, the terms "treatment" or "therapeutic use" as used herein refer to any and all uses of the presently claimed compositions that remedy a disease state, condition, or symptoms, or which prevent, hinder, retard, or reverse the progression of symptoms, conditions, or diseases discussed herein.

Effective amounts of ODF regulatory region constructs, delivery vehicles containing such constructs, agonists, and antagonists, and treatment protocols, can be determined by conventional means. For example, the medical practitioner can commence treatment with a low dose in a subject or patient in need thereof, and then increase the dosage, or systematically vary the dosage regimen, monitor the effects thereof on the patient or subject, and adjust the dosage or treatment regimen to maximize the desired therapeutic effect. Further discussion

of optimization of dosage and treatment regimens can be found in Benet et al., in *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, Ninth Edition, Hardman *et al.*, Eds., McGraw-Hill, New York, (1996), Chapter 1, pp. 3-27, and L.A. Bauer, in *Pharmacotherapy, A Pathophysiologic Approach*, Fourth Edition, DiPiro et al., Eds., Appleton & Lange, Stamford, Connecticut, (1999), Chapter 3, pp.21-43, and the references cited therein, to which the reader is referred.

Viral vector-mediated gene transfer has been used successfully in mouse models and human clinical trials. See Fujiwara et al., *Cancer Research*, 54:2287-2291 (1994), and Roth et al., *Nature Medicine*, 2:985-991 (1996). Mountain, *TIBTECH*, 18:119-128 (2000) discusses recent examples of gene therapy with clinical benefit progressing to Phase II clinical studies using cationic lipids, adenovirus, retrovirus, and adeno-associated virus vectors, as well as naked DNA. Cavazzana-Calvo et al., *Science*, 288:669-672 (2000) reported that gene therapy using a retroviral vector was able to provide full correction of human severe combined immunodeficiency (SCID)-X1 disease phenotype, including clinical benefit. Ueki et al., *J. Clin. Invest.*, 105(10):1437-1445 (2000) reported the successful use of adenovirus-mediated gene therapy to restore insulin sensitivity in mice having a homozygous disruption of insulin receptor substrate-1. Morishita et al., *Biochem. Biophys. Res. Commun.*, 273(2):666-674 (2000) reported that systemic administration of HVJ viral coat-liposome complex containing a human insulin vector decreased glucose levels in diabetic mice, accompanied by the detection of human insulin in liver and spleen. Anderson (*Nature Medicine*, 6(8):862-863 (2000)) has noted that gene therapy has also recently achieved success in the treatment of hemophilia with an adeno-associated viral vector (Kay et al., *Nature Genetics*, 24:257-261 (2000); cardiovascular disease with naked plasmid DNA (Isner et al., *J. Clin. Invest.*, 103:1231-1266 (1999); and cancer therapy using an oncolytic adenovirus (Khuri et al., *Nature Medicine*, 6(8):879-885

(2000). Recent U.S. Patents claiming methods of gene therapy include Nos. 6,080,728 and 6,087,164.

The following examples illustrate various aspects of the present invention, but should not be construed to limit the same.

Example 1

Characterization of the Human ODF Regulatory Region Using 5′ Deletion Constructs

To determine which areas of the presently disclosed ODF regulatory region are required for basal expression, a series of ODF regulatory region 5′ deletion constructs was prepared. Each construct contained the reporter gene beta-galactosidase. See Figure 2. The 4603 bp ODF regulatory region fragment was excised from the pSPORT1 vector, *supra*, with *Sna*BI and *Sal*I, vector restriction sites which flank the *Not*I and *Sst*I sites of the insert. The fragment, SEQ ID NO:11, was then subcloned into the *Sma* I and *Xho* I sites of pβGAL-Basic (Clontech, Palo Alto, CA), and designated pODF4.6βGAL (-4467 to +105).

SEQ ID NO:11 (-4467 to +105)

GGATCCTCTCCGGAGTTCAGCAAAGTGAAACGTCTGTCATAATAATAACGAATGACTTCCTT
TTTCATTTTCATTCATATAGTGAAGTTTTCTAAAGGCTGCATCATGTGCAATATTGTAACAG
AGTAAGTGCAGGACTGAATGTGACTCTATCAGGCCAATTGTAGAGATGTGAAAAAATGTAAA
ACAGTGGCACTTTTCTCCCTACTTTTTTTTTGAAAATCTGTTTTTTTTAAATAAAATAATTTTA
TAAAAGTATTATGAATTATTTATAAAATTATATTAACATTATGTTAACATGCTAATATGGTA
AAATTTTCTGCTTGGAGTTTGAATACACCAAATATTTATAAATATAACTCACACAAATAAAA
CCTCTTTGGTGTTCTCAAAATTTTGAAGAATGTAAAAGGTTTGAAAATTGCTGATCTAGCAA
ATGACTGAACATGAACAGCTATAGTATTTGTACCTGCCCAGCAGTGCAGCAATTCCTTATCC
TTCTCATATCTGCACTTTAATTTTCCTTTGACAAATATCTCTCCCTCCTCTCAGCCCATGAC
ATGAGGTTCACATGGGGTTAACTTAATTCCCTGGCTCAAAGGAAAGGTATTAAATTCAGACT
TGTATCCAACCATTCCTGAAGCTAGACTTAGCCCTATTTTTCAATAACATGAACCAATCAAT
TTTCACATGAGTCCAAAATAATTCTATGTTAATACACTAAGGTACTAGGAAATATAGTTTGA
GAAATGTTGATCCAAACATTGTGTTATTTACAGTGGAGTATTGACATAAACTTTGAATCTTC
AAATATGTTCTGGTGTCTTGGCATCTCTTAATACCTATTAGCTTACAAGGCTTTCACTCAAC
TATTTTATAATTTTGATAATGACTTAATTGATTAGTTGATATATTGTTAAAATAAATATATT
AATGAATTTATGATAAATAAGGCAGATAAATAAGACATGCAATTAGGAAGACATGTTAAACA
AATTGTTATAATAATACAATCACTCTCAGCTTAGGATAGCTCCTGGCCACTTTCTCTCTGGG
TGGTTTTTACTCTGGGAGTAGTTTAAATCATTATCTAGTAGTAGTTTAAAGCATTATCTTTG
CCTAAGAGCTTTCGCTGACTCCCCACATTTGCATTGTACTAAGAGTTTTCTCTGACTCCCCA

26. Use of a compound that modulates expression of osteoclast differentiation factor in the manufacture of a medicament for the treatment of a disease in a human caused by abnormal expression of osteoclast differentiation factor.

27. The use according to claim 26, wherein said disease is bone disease, arthritis, arterial disease, abnormal immune function, abnormal lymph node development, or abnormal T- or B-cell function caused by abnormal expression of osteoclast differentiation factor.

28. The use according to claim 27, wherein said bone disease is malignant bone disease, rheumatoid arthritis, osteoarthritis, elevated bone resorption, osteoporosis, Paget's disease of bone, hypercalcemia of malignancy, expansile osteolysis, or periodontal disease, and said compound is an antagonist of osteoclast differentiation factor expression.

29. The use according to claim 27, wherein said arterial disease is arterial calcification, and said compound is an antagonist of osteoclast differentiation factor expression.

30. The use according to claim 27, wherein said bone disease is osteopetrosis, and said compound is an agonist of osteoclast differentiation factor expression.

31. The use according to any one of claims 24-30, wherein said compound is identified by the method of any one of claims 13-22.

32. The use according to any one claims of 24-31, wherein said human is diagnosed as having a polymorphism or mutation at one or more nucleotide positions in the osteoclast differentiation factor regulatory region in DNA thereof.

45. The method of claim 44, wherein said antagonist is identified by a method according to any one of claims 13-22.

46. A method of treating a human suffering from a symptom, condition, or disease caused by under-expression of osteoclast differentiation factor, comprising administering to said human a pharmaceutically effective amount of an agonist of osteoclast differentiation factor expression.

47. The method of claim 46, wherein said agonist is identified by a method according to any one of claims 13-22.

48. A method of treating a human in need of treatment with an agonist of osteoclast differentiation factor expression, comprising:

(a) determining whether a polymorphism or mutation exists at one or more nucleotide sites in the osteoclast differentiation factor regulatory region in DNA of said human; and

(b) if a polymorphism or mutation exists, administering to said human a pharmaceutically effective amount of an agonist of osteoclast differentiation factor expression.

49. A method of treating a human in need of treatment with an antagonist of osteoclast differentiation factor expression, comprising:

(a) determining whether a polymorphism or mutation exists at one or more nucleotide sites in the osteoclast differentiation factor regulatory region in DNA of said human; and

(b) if a polymorphism or mutation exists, administering to said human a pharmaceutically effective amount of an antagonist of osteoclast differentiation factor expression.

50. The method of claim 48 or 49, wherein said human suffers from a symptom, condition, or disease caused by an abnormal level of expression of osteoclast differentiation factor.

51. A method of modulating bone resorption in a patient in need thereof, comprising administering to said patient a pharmaceutically effective amount of said DNA construct of claim 6 or 7, wherein said protein of interest is osteoclast differentiation factor.

52. A method of modulating bone resorption in a patient in need thereof, comprising administering to said patient a pharmaceutically effective amount of a compound identified by the method of any one of claims 13-22.

53. A method of modulating immune responsiveness in a patient in need thereof, comprising administering to said patient a pharmaceutically effective amount of said DNA construct of claim 6 or 7, wherein said protein of interest is osteoclast differentiation factor.

54. A method of modulating immune responsiveness in a patient in need thereof, comprising administering to said patient a pharmaceutically effective amount of a compound identified by the method of any one of claims 13-22.

55. A kit or package, comprising an isolated nucleic acid fragment comprising the transcriptional regulatory region of the human *odf* gene, or subfragment thereof exhibiting human *odf* gene transcriptional regulatory activity, wherein said fragment or subfragment thereof excludes the *odf* protein coding region.